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(54) Title: ISOLATION OF MAMMALIAN HEMATOPOIETIC STEM CELLS

(57) Abstract

     The present invention relates to a method of purifying mammalian hematopoietic stem cells from a bone marrow cell population. The present invention also relates to a method of separating cycling HSCs from quiescent HSCs in a purified mammalian HSC sample. Further, the invention relates to a method of identifying an agent which is a fluorescent, vital dye which is lipophilic for use in purifying mammalian HSCs. The present invention also relates to a method of transplanting bone marrow in a mammalian host, such as a human, comprising introducing into the host the purified HSCs described herein. The invention further relates to a method of *in vivo* administration of a protein comprising transfecting a purified HSC with a construct comprising DNA which encodes a protein of interest and then introducing the stem cell into the host where the protein of interest is expressed. The present invention also relates to hematopoietic stem cells purified using the methods described herein.

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# ISOLATION OF MAMMALIAN HEMATOPOIETIC STEM CELLS

RELATED APPLICATIONS

This application is a Continuation-in-Part of copending U.S. Patent Application Serial No. 08/471,758,
5   filed June 6, 1995, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Hematopoietic stem cells (HSC) are multipotential cells which reside in the bone marrow and replenish all
10   adult hematopoietic lineages through the lifetime of an animal. Lack of a purified population of stem cells has hampered definitive understanding of the factors which regulate their growth and differentiation. Several schemes for the enrichment of hematopoietic stem cells from murine
15   bone marrow have been developed, but a phenotypically homogeneous population of stem cells has not been identified to date (Visser, J.W., et al., *J. Exp. Med.*, *159*:1576-90 (1984); Pallavicini, M.G., et al., *Exp. Hematol.*, *13*:1173-81 (1985); Spangrude, G.J., et al.,
20   *Science*, *241*:58-62 (1988); Szilvassy, S.J., et al., *Blood*, *74*:930-9 (1989); Jones, R.J., et al., *Nature*, *347*:188-9 (1990); Nicola, N.A., et al., *Blood*, *58*:376-86 (1981); Bertoncello, L., et al., *Exp. Hematol.*, *13*:999-1006 (1985)).
25   The ability to obtain a phenotypically homogeneous population of stem cells is important in achieving greater success with bone marrow transplants (BMTs). For example, autologous BMT could become a candidate for use in leukemic and other cancer therapies if it were possible to
30   definitively remove cancerous cells from the bone marrow.

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Isolation of pure reconstituting HSCs may allow this.
Furthermore, non-autologous BMT may also benefit from HSC
purification, as transplantation of purified HSCs may
ameliorate graft *versus* host disease (GVH) syndromes.  In
5  addition, a homogeneous stem cell population would be
advantageous for hematopoietic stem cell gene therapy.
Previous attempts to infect unmanipulated murine and human
HSCs with recombinant retroviruses have met with mixed
success, presumably at least in part due to the inability
10  of retroviral vectors to integrate into quiescent HSCs,
which are widely held to constitute the majority of the HSC
population.  Thus, purification of HSCs and the ability to
distinguish between quiescent HSCs and cycling HSCs would
have important implications for HSC gene therapy.

15  <u>SUMMARY OF THE INVENTION</u>
       The present invention is based on the discovery that
unusual uptake and fluorescence properties of a dye in
hematopoietic stem cells (HSCs) make it possible to purify
HSCs and isolate a subpopulation of HSCs that are naturally
20  replicating *in vivo*.  Thus, the invention relates to a
method of purifying mammalian hematopoietic stem cells from
a bone marrow cell population.
       In one embodiment, a bone marrow cell sample is
obtained and combined with a fluorescent, lipophilic vital
25  dye which is a substrate for a multiple drug resistant
protein (i.e., mdr protein) under conditions appropriate
for cells to take up the dye.  The term "substrate" is
defined herein as a substance which is removed by mdr.  The
resulting combination is exposed to an excitation
30  wavelength which results in fluorescence of the dye, which
is measured using an emission wavelength.  The amount of
dye contained by each population of cells resolved with the
emission wavelength is determined.  The population of

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nucleated cells which contains the smallest amount of dye
at the emission wavelength is purified, mammalian HSCs.

In another embodiment a bone marrow cell sample is
obtained and combined with a fluorescent, lipophilic vital
5   dye which is a substrate for a mdr protein, under
conditions appropriate for cells to take up the dye.  The
resulting combination is exposed to an excitation
wavelength which results in fluorescence of the dye, which
is measured using two emission wavelengths simultaneously.
10  The amount of dye contained by each population of cells
resolved with the two emission wavelengths is determined.
The population of nucleated cells which contains the
smallest amount of dye at both wavelengths is purified,
mammalian HSCs.

15      The present invention also relates to a method of
separating cycling HSCs from quiescent HSCs in a purified
mammalian HSC sample.  In the method, a purified HSC sample
is obtained and combined with a fluorescent, lipophilic
vital dye which binds DNA and is a substrate for a mdr
20  protein and an inhibitor of the mdr protein under
conditions appropriate for the cells to take up the dye and
the inhibitor.  The amount of dye present in the purified
HSCs is measured, wherein purified HSCs which contain the
smallest amount of dye have a 2n quantity of DNA (i.e.,
25  which refers to the base number of chromosomes in a cell)
and are designated as quiescent HSCs, and purified HSCs
which contain a greater amount of dye have a greater amount
of DNA (i.e. >2n) and are designated cycling HSCs.  This
would also apply to obtaining cycling cells from a
30  population of cells having high mdr activity.  For example,
a heterogeneous population of cells such as bone marrow can
be combined with the dye and the inhibitor.  Cycling and
non-cycling populations are determined as described above.
In this case a heterogenous population of cycling cells are
35  isolated and can be used for infecting with retroviruses.

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Further, the invention relates to a method of identifying an agent which is a fluorescent, vital dye which is lipophilic for use in purifying mammalian HSCs. In one embodiment, a bone marrow cell sample is obtained

5   and combined with the agent under conditions appropriate for the cells to take up the agent. The resulting combination is exposed to an excitation wavelength which results in fluorescence of the agent, which is measured using an emission wavelength and the amount of dye

10  exhibited by each population of cells is determined. If distinct populations of nucleated cells are observed in which one of the population of cells contains the smallest amount of dye and the population is purified, mammalian HSCs, then the agent is a fluorescent vital dye which is

15  lipophilic for use in purifying HSCs.

In another embodiment, a bone marrow cell sample is obtained and combined with the agent under conditions appropriate for the cells to take up the agent. The resulting combination is exposed to an excitation

20  wavelength which results in fluorescence of the agent which is measured using two emission wavelengths simultaneously. The amount of dye contained by each population of cells resolved with the two emission wavelengths is determined. If distinct populations of nucleated cells are observed in

25  which one of the population of cells contains the smallest amount of dye and the population is purified, mammalian HSCs, then the agent is a fluorescent, vital dye which is lipophilic for use in purifying HSCs.

The present invention also relates to a method of

30  transplanting bone marrow in a mammalian host, such as a human, comprising introducing into the host the purified HSCs described herein. The invention further relates to a method of in vivo administration of a protein comprising infecting or transfecting a purified HSC with a construct

35  comprising DNA or RNA which encodes a protein of interest

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and introducing the infected or transfected purified HSC
into the host, in which the protein of interest is
expressed.  In a particular embodiment, exogenous DNA
encoding a protein of interest is infected or transfected
5  into a cycling HSC.

The present invention also relates to hematopoietic
stem cells purified using or obtainable by (obtained by)
the methods described herein.  In one embodiment, the
purified HSCs of the present invention are CD34$^{neg}$.  In
10 another embodiment, the purified HSCs are side population
(SP) cells.  In another embodiment, the purified HSCs have
at least one of the following characteristics:  glycophorin
A$^{neg}$, CD38$^{low/neg}$, CD13$^{neg}$, CD15$^{neg}$, CD19$^{neg}$ and CD20$^{neg}$.

Thus, the present invention allows for the use of
15 purified HSCs in bone marrow transplants.  In addition, the
method of separating cycling HSCs from quiescent HSCs
provides a means of obtaining cycling HSCs, separated from
quiescent HSCs for transfection with a construct encoding a
protein of interest.  As discussed above, recombinant
20 retroviral are unable to integrate into quiescent HSCs.
Thus, the ability to specifically transfect cycling HSCs
will provide greater success in in vivo administration of
proteins to or by HSCs.


BRIEF DESCRIPTION OF THE FIGURES
25    Figure 1A is a graph of Hoechst red versus blue
fluorescence on a linear scale of whole murine normal bone
marrow stained with Hoechst 33342 stain, in which the boxed
region represents the hematopoietic stem cell activity,
which is 0.1% of the total bone marrow cell population.
30    Figure 1B is a graph illustrating the analysis of Sca-
1 and lineage marker staining of whole murine normal bone
marrow.

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Figure 1C is a graph of Hoechst red versus blue fluorescence on a linear scale of whole murine normal bone marrow stained with Hoechst 33342 stain, in which the region indicated in Figure 1B is used as a live gate in conjunction with the gate to exclude red and dead cells.

Figure 1D is a graph illustrating the analysis of Sca-1 and lineage marker staining of whole murine normal bone marrow cells which fall exclusively into the boxed region indicated in Figure 1A.

Figure 1E is a graph of Hoechst red versus blue fluorescence on a linear scale of Sca-1 enriched murine normal bone marrow stained with Hoechst 33342 stain, in which the specific side region indicated in Table 2 is delineated.

Figure 2A is a graph of Hoechst red versus blue fluorescence on a linear scale of whole bone marrow stained with Hoechst 33342.

Figure 2B is a graph of Hoechst red versus blue fluorescence on a linear scale of whole bone marrow stained with Hoechst 33342 in the presence of Verapamil.

Figure 3A is a graph of Hoechst red versus blue fluorescence on a linear scale of purified HSCs restained with Hoechst 33342 in the presence of verapamil.

Figure 3B is a graph of propidium iodide (PI) versus cell number of purified HSC stained with PI.

Figure 4A is a graph of Hoechst red versus blue fluorescence on a linear scale of human whole bone marrow cells stained with Hoescht 33342 stain.

Figure 4B is a graph of Hoechst red versus blue fluorescence on a linear scale of human cord blood cells stained with Hoescht 33342 stain.

Figure 5A is a graph of Hoechst red versus blue fluorescence on a linear scale of porcine bone marrow cells stained with Hoescht 33342 stain.

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Figure 5B is a graph of Hoechst red versus blue fluorescence on a linear scale of Rhesus bone marrow cells stained with Hoescht 33342 stain.


## DETAILED DESCRIPTION OF THE INVENTION

5    The invention relates to a method of purifying mammalian hematopoietic stem cells (HSCs) from a bone marrow cell population.

In one embodiment, a bone marrow cell sample is obtained and combined with a fluorescent, lipophilic vital

10  dye which is a substrate for a multiple drug resistant protein (i.e., mdr protein) under conditions appropriate for cells to take up the dye. The term "substrate" is defined herein as a substance which is removed from a cell by mdr. The resulting combination is exposed to an

15  excitation wavelength which results in fluorescence of the dye, which is measured at an emission wavelength and the amount of dye exhibited by each population of cells is determined. The population of nucleated cells which contains the smallest amount of dye at the emission

20  wavelength is purified, mammalian HSCs.

In another embodiment, a bone marrow cell sample is obtained and combined with a fluorescent, lipophilic vital dye which is a substrate for a mdr protein, under conditions appropriate for cells to take up the dye. The

25  resulting combination is exposed to an excitation wavelength which results in fluorescence of the dye, which is measured using two emission wavelengths simultaneously. The amount of dye contained by each population of cells resolved with the two emission wavelengths is determined.

30  The population of nucleated cells which contains the smallest amount of dye at both wavelengths is purified, mammalian HSCs. As described herein, the method of purifying HSCs has been used to purify side population (SP) cells from four types of mammals: murine, human, monkey and

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porcine. Characterization of the murine SP cells indicate
they are purified HSCs. Further characterization of the
human, monkey and porcine SP cells will confirm they are
purified HSCs. Applicants have shown that the purified

5    HSCs described herein successfully repopulated stem cell
activity in lethally irradicated recipients, providing 1000
fold enrichment of HSC activity in mouse bone marrow. In
addition, the HSCs of the present invention have been shown
to allow survival of lethally irradiated recipients.

10       Thus, the present invention relates to a method for
the isolation or purification of mammalian HSCs, and is
based on the use of FACS analysis of bone marrow cells
stained with a fluorescent vital dye. Starting with
untreated bone marrow, 1,000 fold enrichment of *in vivo* HSC

15   reconstitution activity can be consistently achieved. The
purification strategy has also led to a method for
separating cycling from quiescent stem cells, which will
facilitate efficient gene transfer into hematopoietic stem
cells.

20       The dye which can be used in the method for purifying
HSCs is a fluorescent, vital dye which is a substrate for a
mdr protein. Preferably, the dye is also lipophilic. In
the method of separating cycling HSCs from quiescent HSCs,
the dye must also be a DNA binding dye. In some instances,

25   one dye can be used in the method for purifying HSCs and in
the method for separating cycling HSCs from quiescent HSCs.
For example, as described in Examples 1 and 3, Hoechst
33342 dye (Hoechst dye) can be used in both the method for
purifying HSCs and the method of separating cycling HSCs

30   from quiescent HSCs. Although the method of the present
invention is exemplified using Hoechst dye (Example 1),
other dyes can be used to practice the methods of the
present invention. For example, Rhodamine 123 might be
used to practice the invention, particularly in the

35   embodiment in which purified HSCs are isolated.

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Hoechst 33342 is a fluorescent DNA-binding dye useful for flow cytometry analysis. The dye is readily taken up by live cells, in which it binds to DNA. The quantity of Hoechst 33342 fluorescence relates to DNA content in a cell

5   and, therefore, is an indicator of cell cycle. However, as described in Example 1, when Hoechst dye was used to examine DNA content in murine bone marrow cells by standard methods, a complex fluorescence pattern was observed. When dye fluorescence was observed simultaneously at two

10  emission wavelengths (the red and the blue), several distinct populations could be resolved. These populations are shifted relative to each other predominately with regard to fluorescence on the Hoechst "red" axis (Fig. 1A). Analysis of hematopoietic cells in this staining profile

15  indicated that one population of cells, boxed in Figure 1A, was predominately Sca-1+lin$^{low}$ (Fig. 1D). That is, 75% of the cells from the boxed region in Fig. 1A expressed Ly-6A.2 (Sca-1+), and a low level of six lineage antigens (detected by a cocktail of antibodies against B220, Gr-1,

20  Mac-1, CD4, CD5, and CD8). These cell surface characteristics have previously been identified on murine hematopoietic stem cells by Applicants and others (Spangrude, G.J., et al., *Science*, 241:58-62 (1988); Uchida, N. and Weissman, I.L., *J. Exp. Med.*, 175:175-84

25  (1992)). Characterization of the purified human SP described herein indicate that the purified HSCs are: glycophorin A$^{neg}$, CD38$^{low/neg}$, CD13$^{neg}$, CD15$^{neg}$, CD19$^{neg}$, CD20$^{neg}$, and CD34$^{neg}$. Characterization of purified HSCs in mouse indicate that the purified mouse HSCs are: c-kit$^{pos}$, Sca-1$^{pos}$,

30  Gr-1$^{neg/low}$, Mac-1$^{neg/low}$, CD4$^{neg/low}$, CD8$^{neg}$, B220$^{neg}$, CD5$^{neg}$, CD43$^{pos}$, CD45$^{pos}$, M169$^{pos}$, AA4$^{neg/low}$, class I MHC$^{pos}$, class II MHC$^{neg}$, Rhodamine 123$^{low}$, and Wheat germ agglutinin (WGA)$^{pos}$. This population, referred to as the side population, or SP, represents 0.1% of total bone marrow. These SP cells were

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also a sub-population (approximately 5%) of the total Sca-1+,lin$^{low}$ cells in the bone marrow.

     To examine the functional properties of these cells, long term bone marrow transplantation experiments were
5   performed, as described in Example 2.  A competitive repopulation assay, in which defined numbers of purified cells (i.e., SP cells) were transplanted into lethally irradiated recipients along with unfractionated but distinguishable whole bone marrow cells (i.e.,
10  unfractionated cells), was used.  In this semi-quantitative method, the relative stem cell activity of sorted or SP cells versus unfractionated cells is assessed by determining the percentage of peripheral blood cells descended from each of the two input populations in the
15  bone marrow transplant recipients (Harrison, D.E., *Blood*, *55*:77-81 (1980); Harrison, D.E., et al., *Proc. Natl. Acad. Sci. U.S.A.*, *85*:822-826 (1988)).  It is generally accepted that at four months post-transplant, the majority of peripheral blood cells are derived from long term
20  reconstituting stem cells introduced in the transplant instead of from committed progenitors.  In addition, differentiated cells and progenitors in the unfractionated competitor bone marrow rescue recipients from the otherwise lethal irradiation.  This allows the long term multi-
25  lineage repopulating functions of the input HSC to be examined separately from short-term activities.

     As described in Example 2, in competitive repopulation assays with the SP cells, average enrichments of HSC activity of approximately 1,000 fold over multiple
30  experiments were achieved (Table 1).  Since the SP cells represent 0.1% of the total bone marrow, this indicates that most if not all reconstituting cells in normal mouse bone marrow reside in the SP fraction.  Moreover, competitive repopulation experiments with other Hoechst-

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stained populations shown in Fig. 1A, revealed almost no
additional stem cell activity.  See Table 2.

Having established by competitive repopulation that
stem cell activity fell into the SP region, the ability of
5   that population of cells to protect recipients from lethal
irradiation (i.e., radioprotection) was investigated.  As
shown by data from three separate experiments in Table 3,
approximately 150 purified cells protected 50% of the
recipients from lethal irradiation.  That is, the presence
10  of approximately 150 purified cells in the lethally
irradiated recipients resulted in survival of 50% of the
recipients.

It was of interest to account for the unusual Hoechst-
staining properties of the hematopoietic stem cells.
15  Since it is known that several vital dyes are actively
pumped out of the cells by the mdr protein (i.e.,
p-glycoprotein) (Chaudhary, P.M. and Roninson, L.B., Cell,
66:85-94 (1991)), the possibility that the exceptionally
low Hoechst fluorescence was due to higher mdr activity in
20  murine hematopoietic stem cells was investigated.  As
described in Example 3, bone marrow stained with Hoechst
33342 in the absence or presence of the drug verapamil, an
inhibitor of mdr, demonstrates that the stem cell
population is visible (i.e., able to be isolated) in the
25  absence of verapamil (Fig. 2A, Fig. 2B).

Based upon the ability of verapamil to block the
efflux of Hoechst dye, it was postulated that separation of
replicating stem cells from quiescent stem cells is
possible.  As discovered and described herein, this has
30  been shown to be correct, and can further be used to
separate replicating stem cells from quiescent stem cells.
That is, as shown herein, in the presence of verapamil, the
efflux activity of Hoechst dye is blocked, and a higher
concentration of Hoechst dye is retained in the cells.  In
35  cell populations which do not contain high amounts of mdr

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activity, Hoechst dye reflects DNA content and has commonly
been used in cell cycle analysis.  Cycling cells have a
greater amount of DNA than quiescent cells.  Thus, a
greater amount of dye will be present in cycling HSCs
5    resulting in greater fluorescence of the dye present in the
cycling HSCs when the dye is excited at the appropriate
wavelength.  Conversely, a lower amount of dye will be
present in quiescent cells resulting in lower fluorescence
of the dye present in quiescent HSCs when the dye is
10   excited at the appropriate wavelength.  Thus, measuring the
relative amount of fluorescence of a dye in purified HSCs
allows for separation of cycling HSCs from quiescent HSCs
(Techniques in Cell Cycle Analysis, ed. Gray, J.W. and
Darzynkiewicz, Humana Press, Clifton, NJ (1987)).
15       Accordingly, as described in Example 4, HSCs were
purified (sorted) on the basis of low Hoechst staining, to
produce SP cells as described herein.  The sorted or SP
cells were restained with Hoechst dye in the presence of
verapamil.  Cell-cycle analysis was performed as described
20   in Example 1 and the number of stem cells in $S-G_2M$ (i.e.,
cycling HSCs) was found to range between 1 and 3% of the
purified cells (Fig. 3A).  This figure correlates well with
the number shown to be in $S-G_2M$ by propidium iodide
staining of the purified cells (Fig. 3B).  Stem cells in
25   $G_0-G_1$ (i.e., quiescent HSCs) made up the rest of the
purified cells.
         With a view to using cycling stem cells as targets for
gene transfer, such as retroviral-mediated gene transfer,
the relative engraftment potential of stem cells that were
30   in $G_0-G_1$ versus $S-G_2M$ was compared.  These subsets of the SP
population were purified by flow cytometry using the
verapamil-blocking strategy for cell cycle analysis
described in Example 1, and tested in the competitive

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repopulation assay described in Example 2. Table 4 shows
the results from the experiment.

Thus, the present invention also relates to a method
of separating cycling HSCs from quiescent HSCs in a
5      purified stem cell sample. In this method, a purified HSC
sample is combined with two substances: a fluorescent,
lipophilic vital dye which binds DNA and is a substrate for
a mdr protein and an inhibitor of the mdr protein. This is
carried out under conditions appropriate for the cells to
10     take up the dye and the inhibitor. The amount of dye
present in the HSCs is subsequently measured or observed
for differences in intensity of fluorescence. The extent
to which the dye is contained by a cell is in proportion to
the quantity or concentration of DNA in the cell.
15     Quiescent HSCs contain less or a lower concentration of,
DNA than do cycling HSCs and, therefore, quiescent cells
take up less dye than is taken up by cycling HSCs.
Quiescent HSCs can be distinguished from (i.e., separated
from) cycling HSCs on the basis of the quantity or
20     concentration of the vital dye present. Those which
contain a lesser quantity or concentration of dye are
quiescent HSCs and those which contain a larger quantity or
concentration of dye are cycling HSCs. This would also
apply to obtaining cycling cells from a population of cells
25     having high mdr activity. For example, a heterogeneous
population of cells such as bone marrow can be combined
with the dye and the inhibitor. Cycling and non-cycling
populations are determined as described above. In this
case a heterogenous population of cycling cells are
30     isolated and can be used for infecting with retroviruses.
The invention further relates to a method of
identifying an agent which is a fluorescent, vital dye for
use in purifying hematopoietic stem cells. In one
embodiment, a bone marrow cell sample is obtained and
35     combined with the agent under conditions appropriate for

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the cells to take up the agent. The resulting combination
is exposed to an excitation wavelength which results in
fluorescent of the agent, which is measured using an
emission wavelength. The amount of dye contained by each
5  population of cells resolved with the emission wavelength
is determined. If distinct populations of nucleated cells
are observed in which one of the population of cells
contains the smallest amount of dye and the population is
purified, mammalian HSCs, then the agent is a fluorescent
10  vital dye which can be used in purifying hematopoietic stem
cells.

In another embodiment, an agent is identified by
combining a bone marrow cell sample and an agent to be
assessed, under conditions appropriate for the cells to
15  take up the agent. The resulting combination is exposed to
an excitation wavelength which results in fluorescence of
the dye. The dye fluorescence is measured using two
emission wavelengths simultaneously, so that distinct
populations of live bone marrow cells are resolved on the
20  basis of fluorescence. Dye uptake is assessed in each
population of cells. If distinct populations of cells are
observed in which one of the population of cells contains
the least amount of dye at both wavelengths and the
population is purified, mammalian HSCs, then the agent is a
25  fluorescent, vital dye which is for use in purifying HSCs.

The stem cell purification strategy described herein
can be used with any suitable mammalian (e.g., vertebrate)
species. Applicants have shown that SP cells can be
purified from four types of mammals: mice, humans, monkeys
30  and pigs. As described in Example 5, adult human bone
marrow cells and human cord blood cells have been stained
with Hoechst 33342 and a staining pattern remarkably
similar to that which was observed for murine cells was
observed for these cells. The frequency of cells in the
35  human side population is also approximately 0.03% (range

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0.01% to 0.05%). As described in Example 6, HSCs from porcine bone marrow and monkey bone marrow were purified by using the method of the present invention. Further characterization of the HSCs purified from monkeys, which

5   is described in Example 7, indicates that greater stem cell activity is in the SP cells which are CD34⁻ˢ.

Obtaining a bone marrow cell sample from a mammal for use in the methods of the present invention can be achieved using routine methods known to those of skill in the art.

10  For example, as described in Example 1, the bone marrow cells can be obtained by extracting a cell suspension from the femurs and/or tibias of the mammal, passing the cells through an orifice (e.g., an 18 gauge needle) and pelleting the cells by centrifugation.

15      The HSCs for use in the methods of the present invention can be obtained from any suitable mammalian source such as rodent (e.g., rats, mice), primate, dog, pig, cat, monkey, and/or human sources.

In the method of separating cycling and quiescent

20  purified HSCs, the purified HSCs can be obtained using any suitable method for purifying HSCs. For example, the method for purifying HSCs as described herein can be used. Alternatively, a method of purifying HSCs based on cell surface markers can be used (Visser, J.W., et al., J. Exp.

25  Med., 159:1576-90 (1984); Pallavicini, M.G., et al., Exp. Hematol., 13:1173-81 (1985); Spangrude, G.J., et al., Science, 241:58-62 (1988); Szilvassy, S.J., et al., Blood, 74:930-9 (1989); Jones, R.J., et al., Nature, 347:188-9 (1990); Nicola, N.A., et al., Blood, 58:376-86 (1981);

30  Bertoncello, L., et al., Exp. Hematol., 13:999-1006 (1985)).

The excitation wavelength used in the method of purifying HSCs, is a suitable wavelength which will excite the particular dye chosen to a measurable extent. For

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example, in the embodiment in which the Hoechst dye is used
to purify HSCs, an appropriate excitation wavelength is
from about 250 nm to about 450 nm, and in a particular
embodiment, is about 350 nm.  Hoechst dye emission can be
5    detected at a range of wavelengths, from about 400 nm to
about 700 nm, and in a particular embodiment, about 600 nm.
In another embodiment about 450 nm and about 650 nm can be
used simultaneously to detect Hoechst dye emission.

     In the method of purifying HSCs, the fluorescence of
10   the dye can be measured at one emission wavelength.
Alternatively, two emission wavelengths can be used
simultaneously to measure fluorescence of the dye.
Suitable emission wavelengths are those which will measure
the fluorescence of the dye chosen so that distinct
15   populations of live bone marrow cells are resolved.  For
example, in the embodiment in which the Hoechst dye is used
to purify HSCs, the fluorescence of the Hoechst dye is
measured at two wavelengths using a 450 band pass (BP) 20
and a 675 edge filter long pass (EFLP) optical filter.  As
20   indicated by the graph of Figure 1A, the fluorescence of
the Hoechst dye can also be measured using only the red
emission wavelength (i.e., 675 nm) to obtain purified HSCs.
This was a surprising result since normally 450 nm emission
wavelength is used with Hoechst dye because that is its
25   peak emission wavelength.

     The amount of dye used in the methods of the present
invention functionally will generally be from about 1 $\mu$g/ml
to about 20 $\mu$g/ml dye, preferably from about 5 $\mu$g/ml to
about 15 $\mu$g/ml dye and in particular, about 5 $\mu$g/ml dye.
30        The staining time with the dye (i.e., the length of
time cells are exposed to dye) varies depending on the
temperature at which staining is to occur and the dye
concentration in the methods of the present invention.
Thus, staining can occur overnight or over a number of days
35   at the appropriate temperature.  In particular, the

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staining time with the dye can be from about 30 minutes to
about 180 minutes, preferably between about 60 minutes to
about 120 minutes.  In a particular embodiment of the
method for purifying HSCs, the staining time with the
5   Hoechst dye is about 90 minutes.  In a particular
embodiment of the method for separating cycling and
quiescent purified HSCs, the staining time with the Hoechst
dye is about 60 minutes.

The temperature at which staining with the dye can be
10  carried out is from about 4°C to about 45°C, preferably
about 15°C to about 45°C, and in particular, about 37°C in
the methods of the present invention.  The temperature at
which staining with the dye can be carried out can also be
room temperature (i.e., about 25°C).

15      In a particular embodiment of the present method of
purifying HSCs, 5 μg Hoechst dye is used to stain a bone
marrow cell population and, thus, the HSCs it contains, for
90 minutes at 37°C.  In a particular embodiment for the
method of separating cycling HSCs from quiescent HSCs, 10
20  μg Hoechst dye is used to stain the murine bone marrow cell
population for 60 minutes at 37°C.  In another embodiment,
human and monkey bone marrow were stained for 120 minutes
at 37°C.  In a further embodiment, porcine bone marrow was
stained for 90 minutes at 37°C.

25      In the method of separating cycling HSCs from
quiescent HSCs, an inhibitor of the mdr protein is a
substance or agent which interferes with the activity of
the mdr protein in the HSCs.  That is, an inhibitor of the
mdr protein is a substance or agent which interferes with
30  the ability of the mdr protein to remove the dye from the
HSCs.  Inhibitors of the mdr protein include verapamil,
antibodies directed against mdr (i.e., anti-multiple drug
resistant protein antibody), reserpine, PAK-104P,
vincristine and SDZ PSC 833.  The term "multiple drug
35  resistant protein" as used herein includes the multiple

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drug resistant (mdr) protein and proteins which exhibit mdr-like activity (i.e., an mdr-like efflux of a dye from a HSC). For example, analogs or derivatives of the mdr protein, are included in the term "multiple drug resistant
5 protein".

In the method of separating cycling HSCs from quiescent HSCs, a low amount or quantity of DNA (i.e., 2n DNA or base number of chromosomes in a cell) in HSCs indicates the presence of quiescent HSCs (i.e., HSCs cells
10 in the $G_0$-$G_1$ phase) and a high amount or quantity of DNA (i.e., >2n DNA) in HSCs indicates the presence of cycling HSCs (i.e., HSCs cell in the S-$G_2$M phase).

The HSCs obtained by the method of the present invention can be used in a variety of ways. For example,
15 the HSCs of the present invention can be transplanted into a host (e.g., mammal, particularly human) in need of a bone marrow transplant (e.g., an irradiated host or a host undergoing chemotherapy). In addition, the HSCs of the present invention can be used to treat diseases or
20 conditions in which an individual needs bone marrow cells. The present invention further relates to a method of providing a host with purified HSCs comprising the step of introducing into the host the purified HSCs described herein. In particular embodiments, cycling HSCs or
25 quiescent HSCs are introduced into the host.

The methods of the present invention can be used for in vivo administration of protein by transfecting or infecting purified HSCs with recombinant vectors or constructs comprising DNA which encodes a protein of
30 interest. In particular embodiment, cycling or quiescent purified HSCs are transfected or infected with recombinant constructs comprising DNA or RNA which encodes a protein of interest. Previous attempts to infect unmanipulated HSCs with recombinant retroviral vectors have met with mixed

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success, presumably at least in part due to the inability
of vectors to integrate into quiescent HSCs, which as shown
herein constitute the majority of the HSCs population.
Thus, the method of separating cycling HSCs from quiescent
5    HSCs allows for greater success in infecting HSCs with
recombinant retroviral vectors.  The "administration of
protein" by definition includes the delivery of a
recombinant HSC which expresses a protein in vivo.  For
example, a purified cycling HSC containing a vector,
10   wherein the vector contains a DNA or RNA sequence which
expresses a protein of interest can be administered to a
host under conditions in which the protein of interest is
expressed in vivo (see e.g., United States patent Number
5,399,346 which is herein incorporated by reference).
15        Weissman, et al. have worked with Sca-1$^{positive}$, lin$^{negative}$,
and Thy-1$^{low}$ cells, to characterize murine hematopoietic
stem cells.  However, it has become increasingly clear that
this population is not homogeneous for stem cell activity
(Li, C.L. and Johnson, G.R., "Rhodamine 123 reveals
20   heterogeneity within murine Lin-, Sca-1+ hemopoietic stem
cells", J. Exp. Med. 175:1443-1447 (1992); Spangrude, G.J.
and Johnson, G.R., "Resting and activated subsets of mouse
multipotent hematopoietic stem cells", Proc. Natl. Acad
Sci. USA 87:7433-7437 (1990)).  These cells can be
25   subfractionated with the use of the vital dye Rhodamine-
123, where the 5-10% of cells that contain the lowest
amount of the dye after staining have most if not all of
the long-term-reconstituting activity of the population.
        Fleming et al., J. Cell. Biol., 122:897-902 (1993)
30   have compared the reconstitution potentials of Hoechst
33342-sorted $G_0$-$G_1$ and S-$G_2$M fractions of Thy1.1$^{lo}$Lin-$^{lo}$ Sca-
1+ stem cells.  They observed 18% of the cells in S-$G_2$M,
and reported a lower reconstitution capacity of this sub-
set of cells.  However, as shown here, Hoechst fluorescence

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in HSC reflects mdr-like efflux activity, not DNA content,
unless the efflux activity is blocked with inhibitors such
as verapamil. Therefore, the Hoechst content of their HSC
would be lower than other cells in their population, and
5    even cycling HSC would fall into their $G_0$-$G_1$ peak (and
perhaps below it). Therefore, the reconstitution potential
of the cycling fraction was probably underestimated. They
also examine the cell cycle profile of the Rh123$^{low}$ sub-set
of Thy1.1$^{lo}$ Lin-/$^{lo}$Sca-1+ cells by propidium iodide staining,
10   and find approximately 3% of these cells are in S-$G_2$M.
This figure correlates well with the number of our SP cells
in S-$G_2$M, and it is expected that these populations are
qualitatively extremely similar.

The ability to obtain populations of cycling stem
15   cells also has important implications for hematopoietic
stem cell therapy, including administration of the cells,
as obtained by the present method as modified, such as by
the introduction of an exogenous gene encoding a protein of
interest (e.g., a therapeutic protein). Previous attempts
20   to infect unmanipulated murine and human HSCs with
recombinant retroviruses have met with mixed success,
presumably at least in part due to the inability of
retroviral vectors to integrate in quiescent cells. As
shown here, only 1-3% of HSC are in cycle. The
25   purification of the rare population of stem cells that are
naturally replicating *in vivo* provides an alternative to
transduction strategies based on attempts to influence the
cell cycle status of stem cells.

The invention is further illustrated in the following
30   examples.

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## EXEMPLIFICATION

Example 1:   Characterization of Hoechst 33342 fluorescence
on whole murine bone marrow.

### Preparation of Hoechst 33342-stained Murine Bone Marrow

5       Murine bone marrow was extracted from the femurs and
tibias of C57B1/6 mice, a single cell suspension was made
by passage of the bone marrow through an 18 gauge needle,
and the cells were pelleted by centrifugation.  The bone
marrow cells were resuspended at $10^6$ cells per ml in pre-
10   warmed DMEM contain 2% fetal calf serum, 1mM HEPES, 50
units/ml Penicillin, 50 μg/ml Streptomycin, and 5μg per ml
Hoechst 33342 (Sigma) and incubated for 90 minutes at 37°C.
The resolution of these populations is sensitive to the
staining time and the Hoechst dye concentration (Elwart,
15   J.W. and Dormer, P., Cytometry, 11:239-43 (1990)).

        After Hoechst staining, cells are maintained at 4°C
until FACS analysis.  Any antibody staining or manipulation
is performed at 4°C following the Hoechst stain.  After the
final manipulation, the bone marrow cells were resuspended
20   in HBSS+ containing 2μg/ml propidium iodide (PI).  The
addition of PI did not affect the Hoechst staining profile,
but allowed exclusion of dead cells as described below.

### Flow Cytometer Set-Up

        Analysis and sorting were performed on a dual laser
25   FACStar-plus or Facsvantage (Becton Dickenson).  The
Hoechst dye was excited by the first Argon laser at 350nm
and its fluorescence was measured at two wavelengths using
a 450 band pass (BP) 20 and a 675 edge filter long pass
(EFLP) optical filter (Omega Optical, Brattleboro VT).  A
30   610 dichroic mirror short pass (DMSP) was used to separate
the emission wavelengths.  A 640 EFLP with 640 DMLP have

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also been used with an Enterprise laser (Coherent).
Propidium iodide (PI) fluorescence was also measured
through the 675 EFLP (having been excited at 350 nm).
Hoechst "blue" represents the 450 BP filter, the standard
5   analysis wavelength for Hoechst 33342 DNA content analysis.
Cells positive for PI were seen on the far right of the
Hoechst "red" (675 EFLP) axis shown, and excluded.  Both
Hoechst blue and red fluorescence are shown on a linear
scale.  Optimal cvs off of the first laser are necessary to
10  finely resolve the stem cell population.  The second Argon
laser at 488 nm was used to excite standard fluorochromes
(e.g., fluorescein or phycoerythrin if necessary).  No
cross compensation was necessary.  The gating on forward
and side scatter was not stringent, only erythrocytes and
15  debris were excluded.  Re-analysis of sorted populations
showed purity greater than 98%.


Flow Cytometry Profile: Identification of the Side
Population (SP) Cells
    The Hoechst 33342-stained murine bone marrow is placed
20  on the flow cytometer.  Since the Hoechst fluorescence is
analyzed on a linear scale, optimal cvs are obtained with a
relatively low sample differential, but if the cells are
resuspended at a sufficiently high concentration, they may
still be run at 3000-5000 cells per second.  The sample can
25  be maintained at 4°C.  Initially, the voltages of Hoechst-
detectors are set so that the bulk identifiable population
is centered when Hoechst-Blue is displayed on the Y axis,
and Hoechst-red is displayed on the X axis.  Red blood
cells show up in the far lower left corner as cells which
30  indicate little or no Hoechst fluorescence.  With the UV
laser in the first position, the red blood cells can be
thresholded out.  On an Enterprise laser, the UV beam is in
the second position, so this is not possible.  Dead cells
fluoresce with propidium iodide, and show up on the far

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right of the profile, as very positive on the red axis.  A
live gate is drawn to include only live nucleated cells on
the basis of the above parameters.  In order to identify
the region containing the stem cells, a sufficient number
5   of data points (i.e., events) must be collected during flow
cytometry analysis (100,000 events, where 10,000 events is
standard).  This allows the small side-population (SP) to
be readily identified, as shown boxed in Figure 1A.  When
this region is purified by fluorescence activated cell
10  sorting (FACs) and when used in transplantation assays,
described below, this region contains all of the stem cell
activity in C57B1/6 mice.  The frequency of cells in this
region is close to 0.1%.


Cell Surface Characterization of the Hoechst "Side
15  Population"

     Antibody staining was performed as follows:  Hoechst-
stained bone marrow was suspended in Hanks Balanced Salt
Solution (HBSS) containing 2% fetal calf serum, 1mM HEPES,
penicillin, and streptomycin (HBSS+) at $10^8$ cells per ml.
20  The antibodies that make up the lineage cocktail were added
at 1/50 to 1/100 dilutions (after being titered for this
cell concentration).  The cocktail is comprised of the
following:  CD4 (GK1.5, Becton Dickenson); CD8 (53-6.7,
Becton Dickenson); CD5 (53-7.3, Pharmingen); B220 (RA3-6B2,
25  Caltag); Mac-1 (M1/70.15, Caltag); Gr-1 (RB6-8C5,
Pharmingen).  The mixture was incubated on ice for 10
minutes, then the bone marrow was washed once in excess
HBSS+ and the cells were pelleted through a serum cushion.
All washes were performed in this manner.  The cells were
30  resuspended in media containing Goat anti-rat antibody
conjugated to phycoerythrin (mouse-serum adsorbed, Caltag),
and incubated for 10 minutes on ice.  After washing, the
cells were resuspended in 1/3 volume rat serum (Cappel) and

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2/3 HBSS+. After 10 minutes on ice, biotinylated Sca-1
antibody (E13 161-7) was added for 10 minutes on ice.
After washing, the cells were stained with avidin-FITC
(Becton Dickenson) for 10 minutes on ice. Alternatively,
5  Goat-anti-rate-FITC may be used to detect the lineage
antibodies, and streptavadin-PE for Sca-1.

        After the final wash, the bone marrow cells were
resuspended in HBSS+ containing 2µg/ml propidium iodide
(PI). The addition of PI did not affect the Hoechst
10 staining profile as shown in Fig. 1A, but allowed exclusion
of dead cells as described above. In sorting experiments,
the bone marrow was sometimes magnetically pre-enriched for
Sca-1 positive cells using the MACS (Miltenyi Biotec) and
streptavidin microbeads. This resulted in a 5 to 10 fold
15 enrichment, and did not affect enrichment data. Cells were
sorted into glass tubes containing 100% fetal calf serum.
An aliquot was removed and reanalyzed to establish high
purity, and cells were washed and counted prior to dilution
for bone marrow transplantation.

20      The Sca/lin profile on whole bone marrow is shown in
Figure 1B with the region shown by us and others to contain
all of the stem cell activity in the mouse indicated. This
region contains between 1-5% of the cells in the bone
marrow after live gating out the red blood cells and dead
25 cells as described above. This number varies depending on
which secondary conjugates are used to detect Sca-1 or the
lineage cocktail.

        If the region indicated in Fig. 1B is used as a live
gate (in conjunction with the gate to exclude red and dead
30 cells), the Hoechst profile is heterogeneous as shown in
Fig. 1C. 5-10% of the cells fall into the side population.
If the region indicated in Figure 1A is used as the SOLE
LIVE GATE on the whole population of murine bone marrow
cells, the sca/lin profile of those cells is shown in
35 Figure 1D. In the example shown, approximately 75% of the

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cells fall into the Sca-1$^{positive}$ lineage$^{negative}$ region shown to
contain all of the stem cells. Reducing the size of the
side population box to avoid contamination from the bulk
population increases the apparent cell surface homogeneity
5    of the SP cells up to about 95%.

This procedure to characterize the cell surface
expression of the Sca-1 and lineage antigens can be
repeated for any marker alone or in combination. The
following characterizations have thus been made for the
10   human SP cells:  glycophorin A$^{neg}$, CD38$^{low/neg}$, CD13$^{neg}$, CD15$^{neg}$,
CD19$^{neg}$, CD20$^{neg}$, and CD34$^{neg}$. The following characterizations
have been made for the murine SP cells:  c-kit$^{pos}$, Sca-1$^{pos}$,
Gr-1$^{neg/low}$, Mac-1$^{neg/low}$, CD4$^{neg/low}$, CD8$^{neg}$, B220$^{neg}$, CD5$^{neg}$, CD43$^{pos}$,
CD45$^{pos}$, M169$^{pos}$, AA4$^{neg/low}$, class IMHC$^{pos}$, class II MHC$^{neg}$,
15   Rhodamine 123$^{low}$ and Wheat germ agglutinin (WGA)$^{pos}$


Example 2:  Long Term Bone Marrow Transplants Using
Hoechst-purified stem cells


Competitive Repopulation Assay Using Hoechst-purified stem
cells
20        To examine the functional properties of the cells
purified in Example 1, long term bone marrow
transplantation experiments were performed. For the
competitive repopulation assay congenic C57B1/6 mouse
strains which differ at the Ly-5 locus were used (Scheid,
25   M.P. and Triglia, D., *Immunogenetics,* 9:423-433 (1979)).
The Ly-5 antigen is found on the surface of all nucleated
peripheral blood cells and the two allelic variants (i.e.,
Ly-5.1 and Ly-5.2) are readily distinguished by specific
monoclonal antibodies. Peripheral blood was collected from
30   transplant recipients at multiple time points and assayed
by flow cytometry for the proportion of the two Ly-5
alleles present in each blood lineage.

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Typically, bone marrow was extracted from femurs and
tibias of 10 male C57B1/6-Ly-5.1 mice (National Cancer
Institute) 6-10 weeks in age and purified as described in
Example 1.  Stem cells were purified on the basis of the
5    combination of the Hoechst side population and Sca-1+lin$^{low}$
fluorescence, as described in Example 1.  This population
was selected as follows:  first, a live gate was defined
using Hoechst red and blue axis to exclude dead cells and
debris.  After collecting 10$^5$ events within this live gate,
10    the SP population is able to be clearly defined.  A new
live gate is established on this population, as shown boxed
in Figure 1A, and the fluorescence of Sca-1 and lineage
markers on SP cells was displayed.  This allows a clear
definition of Sca-1+ Lin$^{low}$ cells, as the bulk of the SP
15    cells stand out immediately as falling into the region
defined in Figure 1B.  A sorting gate is then defined as
cells which fall into both the region boxed in Figure 1A
and in Figure 1B.  Cells are purified by FACs and when they
are reanalyzed, the purity is greater than 98%.
20        These purified cells were counted and mixed with
unfractionated bone marrow cells (i.e., competitor cells)
obtained from two male C57B1/6-Ly-5.2 mice (Jackson
Laboratory), 6-10 weeks in age, and the mixtures were
introduced into female C57B1/6-Ly-5.2 recipient mice (i.e.,
25    recipients) (Jackson Laboratory).  The recipients were 6 to
12 weeks in age, and maintained on acidified water.  The
recipients were irradiated with 1100 rads given in two
doses (620 rads and 480 rads) at least 2 hours apart.
Transplanted cells were given intravenously by retro-
30    orbital injection under methoxyflurane (Pittman Moore)
anesthetic.  Peripheral blood was taken by retro-orbital
puncture under methoxyflurane anesthesia 4 months post-
transplant.  All animal care was in accordance with
institutional guidelines.  Red blood cells were removed and

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the nucleated peripheral blood cells were stained with
biotinylated anti-Ly-5.1 antibody which was detected with
streptavidin-PE (Molecular Probes). Sometimes, the blood
samples were subdivided and co-stained with directly
5   conjugated lineage specific antibodies against Thy-1, B220,
Gr-1, and/or Mac-1 (Pharmingen). These blood samples were
analyzed on the Facstar-plus, or a Facscan (Becton
Dickenson). The anti-Ly-5.2 and anti-Ly-5.1 hybridomas
used were 104.2.1 and A20.1.7 respectively (gifts from D.
10  Pardoll). Other anti-Ly-5.2 and anti-Ly-5.1 hybridomers
are available for use (Spangrude, G.J., et al., *Science,
241*:58-62 (1988)).

The results are shown in Table 1. The first column
displays the number of Hoechst-purified stem cells (HSC).
15  The second column displays the number of competitor cells
introduced (see below). N represents the number of
transplant recipients at the time of analysis (usually the
same as the number initially transplanted) in each group.
The mean is the mean number of HSC-derived (Ly-5.1+)
20  nucleated peripheral blood cells present in recipients 4
months post-transplant, and SD is the standard deviation.
Enrichment is calculated as the mean percentage
contribution Ly-5.1+ cells in the peripheral blood per
purified cell introduced, divided by the mean percentage
25  contribution of Ly-5.2+ cells in the peripheral blood per
unfractionated Ly-5.2 cell introduced. All numbers are
rounded to two significant digits.

As shown in Table 1, the peripheral blood was analyzed
4 months post-transplant, ensuring that most of the cells
30  present were derived from HSC. Furthermore, the
contribution of the SP cells to the total stem cell
activity was maintained at the same level for at least 12
months post transplant. In addition, the SP cells have
been shown to contribute to all major blood lineages.
35  Interestingly, as seen in Table 1, lower does of purified

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SP cells routinely appear to give rise to apparently higher enrichments, suggesting that there is some complex regulation when large numbers of purified stem cells are introduced into recipients.

Table 1. Competitive Repopulation by Hoechst-Purified Stem Cells

| HSC | compet x10⁻³ | n | mean | 8D | enrichment |
|---|---|---|---|---|---|
| 1000 | 200 | 5 | 79 | 11 | 720 |
| 300 | 200 | 4 | 54 | 14 | 780 |
| 100 | 200 | 4 | 43 | 3 | 1500 |
| 1000 | 400 | 4 | 52 | 2 | 400 |
| 300 | 400 | 3 | 26 | 9 | 470 |
| 100 | 400 | 5 | 29 | 15 | 1600 |

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5    <u>Competitive repopulation assay using various regions of the
     hematopoietic stem cell populations in Figure 1E</u>

         Each of the three regions in Figure 1E was purified as
     described in Example 1 and tested for stem cell activity
     using the competitive repopulation assay described above.
10   Bone marrow from C57B1/9-Ly-5.1 mice was magnetically
     enriched for Sca-1 antibody staining cells to afford
     approximately 8-fold enrichment for stem cell activity.
     Sca-1 is known to mark all of the stem cells in this strain
     of mice.

15       The results are shown in Table 2.  The first column
     indicates the region of cells tested.  The second column
     displays the number of purified cells transplanted.  The
     third column displays the number of competitor cells
     introduced.  N represents the number of transplant
20   recipients at the time of analysis (usually the same as the
     number initially transplanted) in each group.  The mean is
     the mean number of nucleated peripheral blood cells derived
     from the fractionated bone marrow present in recipients 4
     months post-transplant, and SD is the standard deviation.
25   Enrichment is calculated as the mean % Ly-5.1$^+$ cells in the
     peripheral blood per purified cell introduced, divided by
     the mean percentage contribution of Ly-5.2$^+$ cells per
     unfractionated Ly-5.2 cell introduced.  All numbers are
     rounded to two significant digits.

Table 2. Competitive Repopulation Assay using Three Regions of the SP Cells.

| Fraction | # cells x 10⁻³ | #5.2 x 10⁻³ | n | % 5.1⁺PBL | SD | Enrichment |
|---|---|---|---|---|---|---|
| SP | 2 | 1000 | 6 | 56 | 5 | 640 |
| SP | 1 | 1000 | 7 | 41 | 5 | 698 |
| SP | 0.5 | 1000 | 7 | 30 | 8 | 960 |
| upper SP | 8.2 | 500 | 5 | 31 | 11 | 28 |
| NOT SP | 20 | 500 | 5 | 8 | 3 | 2 |

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Radioprotective ability of purified stem cells

      Having established average enrichments of HSC activity of approximately 1000 fold with the SP cells in the competitive repopulation assays, the ability of the SP

5 cells to protect recipients from lethal irradiation was investigated.

      Purified HSC (male C57B1/6-Ly-5.1 derived, Hoechst SP., Sca-1+, lin$^{low}$) were introduced into female C57B1/6-Ly-5.2 recipients as described above for the competitive

10 repopulation assay. Results from three independent experiments are shown in Table 3. The number of purified HSCs introduced into the recipient mice are shown in the first column. The number of animals transplanted in each group is shown in the second column. The percentage of

15 animals surviving at least 4 months post-transplantation is shown in the third column. The mean percent Ly-5.1 nucleated peripheral blood cells in the transplant recipients 4 months post-transplant is shown in column four. The standard deviation (SD) is shown in column five.

20 All numbers are rounded to two significant digits.

      As shown by data from three separate experiments in Table 3, approximately 150 purified cells radioprotected 50% of recipients. The mean contribution of the SP cells to hematopoiesis in the peripheral blood of survivors was

25 at least 81%. Although others have observed a higher level of radioprotective activity from their purified populations, the mean contribution of the transplanted stem cells to hematopoiesis in their survivors was considerably lower (Spangrude, G.J., et al., *Science*, 241:58-62 (1988);

30 Fleming, W.H., et al., *J. Cell Biol.*, 122:897-902 (1993); Li, C.L., Johnson, G.R., *Exp. Hematol.*, 20:1309-15 (1992); Spangrude, G.J. and Scollay, R., *Exp. Hematol.*, 18:920-926 (1990)). This may reflect qualitative differences in the purified populations, or possibly differences in animal

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husbandry practices which affect survival after lethal
irradiation.  In addition, the 12-day spleen colony
formation (CFU-s) of this population was measured to be
approximately 1 CFU-s(12) per 35 purified cells injected.

5   This frequency correlates well with the CFU-s(12) forming
frequency of the Rhodamine-123$^{low}$ sub-population of Sca-1+
lin$^{low}$ Thy-1.1$^{low}$ cells (Spangrude, G.J. and Johnson, G.R.,
*Proc. Natl. Acad. Sci. U.S.A.*, 87:7433-7 (1990)).

Table 3.  Radioprotective Ability of Purified Stem Cells

| HSC | n | % survival | mean | SD |
|---|---|---|---|---|
| 25 | 10 | 0 | 0 | - |
| 50 | 10 | 10 | 12 | - |
| 100 | 20 | 25 | 81 | 11 |
| 200 | 10 | 80 | 87 | 10 |
| 500 | 10 | 70 | 89 | 6 |
| 25 | 20 | 4 | 1 | - |
| 50 | 19 | 0 | 0 | - |
| 100 | 29 | 24 | 83 | 18 |
| 200 | 20 | 60 | 89 | 6 |
| 0 | 15 | 0 | 0 | - |
| 100 | 20 | 40 | 85 | 16 |
| 250 | 20 | 45 | 95 | 4 |
| 500 | 20 | 70 | 97 | 1 |
| 1000 | 19 | 68 | 97 | 1 |

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Example 3:  Inhibition of Multiple Drug Resistant protein
with Verapamil

Since it is known that several vital dyes are actively
pumped out of the cells by the multidrug resistance protein
5    (mdr, or p-glycoprotein), the possibility that the
exceptionally low Hoechst fluorescence was due to higher
mdr activity in murine hematopoietic stem cells was
investigated (Chaudhary, P.M. and Roninson, L.B., *Cell,
66*:85-94 (1991)).

10   Whole murine bone marrow was stained for 90 minutes in
5µg/ml Hoechst 33342 as described in Example 1 with 50 µM
verapamil or without verapamil (Sigma).  After staining,
samples were kept on ice until flow cytometry analysis as
described in Example 1.

15   The results, which are shown in Figures 2A (without
verapamil) and 2B (with verapamil), demonstrate that
purified stem cell population cannot be isolated in the
presence of verapamil using the method of the present
invention.  Therefore, it is reasonable to expect that the
20   stem cells are uniquely low in Hoechst fluorescence due to
mdr or an mdr-like mediated efflux of the dye.  This very
high dye efflux activity may be due to a higher level of p-
glycoprotein on the surface of HSC, a higher level of
activity of the p-glycoprotein present, or an mdr-like
25   activity not yet identified.  Antibody studies on the
expression of the product of the MDR1 gene in human
hematopoietic cells suggest that p-glycoprotein is in fact
expressed quite widely, and that up to 65% of bone marrow
cells may express mdr (Chaudhary, P.M. and Roninson, L.B.,
30   *Cell, 66*:85-94 (1991); Drach, D., et al., *Blood, 80*:2729-34
(1991)).  In light of this, a purification strategy based
on functional properties, such as the one described herein,
will likely be more powerful than a scheme based on the
level of mdr cell surface expression (e.g., antibody-
35   based).

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Example 4:   Analysis of the Cell Cycle Status of Purified
Hematopoietic Stem Cells

        Initial purification of the HSC was as described in
Example 1.   After sorting to 98% purity, several thousand
5   sorted stem cells were incubated for 60 minutes at 37°C in
10 ml DMEM containing 2% fetal calf serum, 1mM HEPES,
Penicillin, Streptomycin, 10 $\mu$g per ml Hoechst 33342, and
50 $\mu$M verapamil.   Flow cytometry analysis was as described
in Example 1.   Propidium iodide stain:   Stem cells purified
10   as described above were pelleted by centrifugation and
resuspended in 0.1% NaCitrate, 50 $\mu$g/ml propidium iodide.
After incubation on ice for 10 minutes, the cells were
analyzed by standard flow cytometry procedures using 488 nm
excitation.   The results are shown in Fig. 3A and Fig. 3B.
15       An aliquot of the SP cells was removed and
transplanted as the "1x sorted" group in column 1.   The
rest of the sorted cells were incubated for 60 minutes in
DMEM as described in Figure 1, containing 10 $\mu$g/ml Hoechst
33342 and 50 $\mu$M Verapamil.   These were resuspended in HBSS
20   including 2 $\mu$g/ml of propidium iodide, and sorted on the
flow cytometer as described in Figure 1.   Several thousand
cells were first allowed to pass through the laser without
subfractionation.   These were transplanted as the "2x
sorted" group.   Large differences in the reconstitution
25   potential between the 1x sorted and 2x sorted groups could
have indicated damage of the HSC by the sorting conditions.
The remaining cells were sorted into either 2n ($G_0$-$G_1$) or
>2n (S-$G_2$M) groups, and transplanted as indicated.
        When cell-cycle analysis was performed in this way,
30   the number of cells in S-$G_2$M (i.e., cycling HSCs) was found
to range between 1 and 3% of the purified cells (Fig. 3A).
This figure correlates well with the number shown to be in
S-$G_2$M by propidium iodide staining of the purified cells
(Fig. 3B).   This is much lower than the fraction of cells

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in S-$G_2$M in whole bone marrow (20%), and in other
populations enriched for stem cells (Fleming, W.H., et al.,
J. Cell Biol., 122:897-902 (1993)). This low number of
cells in cycle supports the widely held view that HSC from
5   normal bone marrow are largely quiescent.

With a view to using cycling stem cells as targets for
retroviral-mediated gene transfer, the relative engraftment
potential of stem cells that were in $G_0$-$G_1$ (i.e., quiescent
HSCs) versus S-$G_2$M was compared. These subsets of the SP
10  population were purified by flow cytometry using the
verapamil-blocking strategy for cell cycle analysis
described in Example 1, and tested in the competitive
repopulation assay described in Example 2. Table 4 shows
the results from the experiment.

15  One experiment is shown with the analysis performed at
both 2 months and 11 months post-transplant. The number of
purified or sub-fractionated HSC transplanted per mouse is
shown in the second column (HSC). The number of
unfractionated Ly-5.2 competitor bone marrow cells co-
20  transplanted is indicated in the third column, and the
number of mice at the time of analysis (n) in the fourth
column. The mean shown is the mean percentage of Ly-5.1+
nucleated peripheral blood cells at the time post-
transplant indicated, and SD is the standard deviation.
25  The enrichment is calculated as in Table 1. All numbers
are rounded to two significant digits.

These data demonstrate several points. Firstly, the
contribution of the $G_0$-$G_1$ HSC to the peripheral blood is
almost identical to that of the S-$G_2$M HSC, and both of
30  these populations are virtually identical in activity to
HSC which have not been sub-fractionated (1x and 2xx sorted
HSC). Secondly, a passage of HSCs through the flow
cytometer twice (2x sorted; exciting the fluorescence of a
DNA-binding dye with a UV laser) does not seem to affect

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the capacity of the HSC to repopulate the bone marrow.
Thirdly, the percentage of peripheral blood cells derived
from the sorted cells is almost identical at 2 months and
11 months, reflecting the very long term stem cell activity
5   of all of these populations.

Table 4.  Competitive Repopulation With Cycling and Non-Cycling Cells

| Group | HSC | compet x10$^{-3}$ | n | mean | SD | enrichment |
|---|---|---|---|---|---|---|
| **2 Month** | | | | | | |
| 1x sorted | 400 | 400 | 9 | 44 | 10 | 800 |
| 2x sorted | 400 | 400 | 7 | 56 | 6 | 1300 |
| 2x sorted | 200 | 200 | 6 | 52 | 16 | 1100 |
| $G_0-G_1$ | 400 | 400 | 6 | 47 | 9 | 870 |
| $G_0-G_1$ | 200 | 200 | 7 | 62 | 17 | 1600 |
| $S-G_2M$ | 200 | 200 | 5 | 64 | 2 | 1800 |
| **11 Month** | | | | | | |
| 1x sorted | 400 | 400 | 7 | 51 | 14 | 1000 |
| 2x sorted | 400 | 400 | 6 | 60 | 8 | 1500 |
| 2x sorted | 200 | 200 | 6 | 56 | 26 | 1300 |
| $G_0-G_1$ | 400 | 400 | 6 | 54 | 7 | 1200 |
| $G_0-G_1$ | 200 | 200 | 7 | 60 | 11 | 1500 |
| $S-G_2M$ | 200 | 200 | 5 | 63 | 20 | 1700 |

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5    Example 5: Characterization of Hoechst 33342 Fluorescence
     on Whole Human Bone Marrow and Human Cord Blood

     Cell surface characterization of human SP cells
          Hematopoietic stem cells from whole human bone marrow
10   and human umbilical cord blood were identified as described
     in Example 1, except as described below.  Human SP cells
     were characterized following the procedure for cell surface
     characterization of the mouse SP cells described in Example
     1.  In some samples, there are some CD34-positive cells at
15   the TOP of the SP region, near the bulk population of
     cells.  But the majority of cells in the SP region in
     several samples of human marrow that we have examined are
     characterized as follows: Glycophorin A$^{neg}$, CD38$^{low}$, CD13$^{neg}$,
     CD15$^{neg}$, CD19$^{neg}$, CD20$^{neg}$ and CD34$^{neg}$.  The human cells run at a
20   lower frequency, closer to 0.03-0.05% in the bone marrow.
     Typically, this bone marrow has been depleted of red blood
     cells and platelets, by a ficoll density centrifugation.
     The results are shown in Figures 4A and 4B.


25   Example 6:  Characterization of Hoechst 33342 Fluorescence
     on Porcine Bone Marrow and Monkey Bone Marrow
          Hematopoietic stem cells from porcine bone marrow and
     monkey bone marrow were identified as described in
     Example 1, except as indicated below.  Porcine bone marrow
30   and Rhesus macaque monkey bone marrow were characterized
     following the procedure for cell surface characterization
     of the mouse SP cells described in Example 1.  In addition,
     fresh bone marrow was depleted of red blood cells in the
     case of Rhesus bone marrow by a ficoll density gradient.
35   Nucleated cells were counted and resuspended at 10$^6$
     nucleated cells per ml in pre-warmed DMEM/10% FCS, 1mM
     HEPES, and 50 units per ml penicillin and 50 $\mu$g per ml
     streptomycin.  Hoechst 33342 dye was added to a

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concentration of 5 μg/ml, and the cells were incubated at
37°C for 120 minutes.  Bone marrow samples were run on the
flow cytometer using the set-up described for the murine
bone marrow.

5       The results are shown in Figures 5A and 5B.  The
arrows indicate the presence of cells in the same region
murine stem cell activity is observed (see Figure 1A).  The
region represents 0.05% to 0.1% of the nucleated cell
population in both of these animal models.  The phenotype

10  of the Rhesus monkey SP cells are as follows:  CD34$^{neg}$,
CD38$^{low/neg}$, CD4$^{neg}$, CD8$^{neg}$, Glycophorin A negative, CD61$^{neg}$, and
CD66$^{neg}$.


Example 7:   Rhesus Long Term Culture Initiating Cell
15  (LTCIC) Limiting Dilution Experiments
        The LTCIC assay is considered by many in the field to
represent the best $in$ $vitro$ assay for HSCs.  As described
below, this assay was performed on hematopoietic stem cells
from Rhesus monkeys.  Hematopoietic stem cells from Rhesus

20  monkeys were identified as described in Example 1.  The
LTCIC assays were performed as described in Sutherland,
H.J., et al., $Proc.$ $Natl.$ $Acad.$ $Sci.$, 87:3584-3588 (1990).
The results are shown in Table 5.


Table 5.  Rhesus LTCIC Limiting Dilution Experiments.

| Unfractionated | Unfract./HO-stained | CD34$^{pos}$/CD38$^{neg}$ | SP$^{+}$/CD34$^{neg}$ |
|---|---|---|---|
| 1/4 x 10$^3$ | 1/5 x 10$^3$ | 1/55 | 1/6 |
| 1/12 x 10$^3$ | 1/15 x 10$^3$ | 1/53 | 1/8 |
| 1/12 x 10$^3$ | 1/8 x 10$^3$ | 1/51 | 1/7 |


        Numbers given represent number of LTCIC forming units
per cell plated in a limiting dilution-type assay.  Bone
marrow from three separate monkeys is shown.  As shown in

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Table 5, the hematopoietic stem cell activity on a per cell
5   basis of the SP cells that are CD34 negative (column 4) is
    significantly higher than the CD34$^{pos}$/CD34$^{neg}$ cells
    (column 3), which are considered in the art to define stem
    cells, from the same animal.  The controls are ·
    unfractionated (column 1) and unfractionated/Hoechst-
10  stained (column 2) marrow.  The unfractionated and CD34$^{positive}$
    numbers of LTCIC are right in the range of LTCIC activity
    found in these populations in the literature.  (Sutherland,
    H.J., et al., *Proc. Natl. Acad. Sci.*, *87*:3584-3588 (1990)).
        Thus, the data indicates that in the monkey, SP cells,
15  which are CD34$^{neg}$, are highly enriched for LTICs.


                            <u>EQUIVALENTS</u>
        Those skilled in the art will recognize, or be able to
    ascertain using no more than routine experimentation, many
20  equivalents to the specific embodiments of the invention
    described specifically herein.  Such equivalents are
    intended to be encompassed in the scope of the following
    claims.

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CLAIMS

We claim:

1.    A method of purifying mammalian hematopoietic stem
      cells from a bone marrow cell sample comprising the
5     steps of:
      a)    obtaining a mammalian bone marrow cell sample;
      b)    combining the cell sample of a) with a
            fluorescent, lipophilic vital dye which is a
            substrate for a multiple drug resistant protein,
10          under conditions appropriate for cells in the
            cell sample to take up the dye;
      c)    exposing the combination produced in b) to an
            excitation wavelength which causes fluorescence
            of the dye;
15    d)    assessing the fluorescence of the dye using an
            emission wavelength,
      wherein nucleated cells which contain the least amount
      of dye are purified mammalian hematopoietic stem
      cells.
20

2.    A method of Claim 1 wherein the dye is Hoechst 33342
      dye, the excitation wavelength is about 350 nm and the
      emission wavelength is from about 650 nm to about 675
      nm.
25

3.    A method of Claim 1 wherein the hematopoietic stem
      cells are human cells.


4.    A method of Claim 3 wherein the hematopoietic stem
30    cells are glycophorin A$^{neg}$, CD38$^{low/neg}$, CD13$^{neg}$, CD15$^{neg}$,
      CD19$^{neg}$, CD20$^{neg}$, and CD34$^{neg}$.


5.    A method of Claim 1 further comprising the step of
      combining the mammalian bone marrow sample with

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antibodies which bind cell surface markers of
hematopoietic stem cells prior to step c).

6.  Purified hematopoietic stem cells obtainable by the
    method of Claim 1.

7.  A method of purifying mammalian hematopoietic stem
    cells from a bone marrow cell sample comprising the
    steps of:
    a)  obtaining a mammalian bone marrow cell sample;
    b)  combining the cell sample of a) with a
        fluorescent, lipophilic vital dye which is a
        substrate for a multiple drug resistant protein,
        under conditions appropriate for cells in the
        cell sample to take up the dye;
    c)  exposing the combination produced in b) to an
        excitation wavelength which causes fluorescence
        of the dye;
    d)  assessing the fluorescence of the dye using two
        different emission wavelengths simultaneously,
    wherein nucleated cells which contain the least amount
    of dye are purified mammalian hematopoietic stem
    cells.

8.  A method of Claim 7 wherein the dye is Hoescht 33342
    dye.

9.  A method of Claim 7 wherein the hematopoietic stem
    cells are human cells.

10. A method of Claim 9 wherein the hematopoietic stem
    cells are glycophorin $A^{neg}$, $CD38^{low/neg}$, $CD13^{neg}$, $CD15^{neg}$,
    $CD19^{neg}$, $CD20^{neg}$, and $CD34^{neg}$.

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11.  A method of Claim 7 wherein the excitation wavelength
     is about 350 nm and the two emission wavelengths are
     about 450 nm and about 675 nm.

5   12.  A method of Claim 7 further comprising the step of
         combining the mammalian bone marrow cell sample with
         antibodies which bind cell surface markers of
         hematopoietic stem cells prior to step c).

10  13.  Purified, mammalian hematopoietic stem cells
         obtainable by the method of Claim 7.

14.  A method of purifying mammalian hematopoietic stem
     cells from a bone marrow cell sample comprising the
15   steps of:
     a)   obtaining a mammalian bone marrow sample;
     b    combining the cell sample of a) with Hoechst
          33342 dye, under conditions appropriate for cells
          in the cell sample to take up the dye;
20   c)   exposing the combination in b) to an excitation
          wavelength of about 350 nm thereby causing
          fluorescence of the dye;
     d)   measuring the fluorescence of the dye using
          emission wavelengths of 450 nm and 675 nm
25        simultaneously;
     e)   determining the amount of dye uptake of each cell
          population of d) using flow cytometry,
     wherein nucleated cells which contain the least amount
     of dye are purified mammalian hematopoietic stem
30   cells.

15.  The method of Claim 14 wherein the hematopoietic stem
     cells are human cells.

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16.   A method of Claim 15 wherein the hematopoietic stem
      cells are glycophorin A$^{neg}$, CD38$^{low/neg}$, CD13$^{neg}$, CD15$^{neg}$,
      CD19$^{neg}$, CD20$^{neg}$, and CD34$^{neg}$.


5  17.   A method of Claim 14 further comprising the step of
         combining the mammalian bone marrow cell sample with
         antibodies which bind cell surface markers of the
         hematopoietic stem cells prior to step c).


10 18.   Purified, mammalian hematopoietic stem cells
         obtainable by the method of Claim 14.


   19.   A method of separating cycling hematopoietic stem
         cells from quiescent hematopoietic stem cells in a
15       purified mammalian hematopoietic stem cell sample
         comprising the steps of:
         a)    obtaining a purified mammalian hematopoietic stem
               cell sample;
         b)    combining the cells of a) with a fluorescent,
20             lipophilic, vital dye which is a substrate for a
               multiple drug resistant protein and binds DNA and
               an inhibitor of the multiple drug resistant
               protein under conditions appropriate for the
               cells to take up the dye and the inhibitor; and
25       c)    measuring the amount of dye present in the
               hematopoietic stem cells,
         wherein purified hematopoietic stem cells which take
         up the smallest amount of dye have a low concentration
         of DNA and are designated as quiescent hematopoietic
30       stem cells, and purified hematopoietic stem cells
         which take up a greater amount of dye have a greater
         amount of DNA and are designated cycling hematopoietic
         stem cells.

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20. A method of Claim 19 wherein the inhibitor of the
    multiple drug resistant protein is selected from the
    group consisting of: verapamil and anti-multiple drug
    resistant protein antibodies.

5

21. A method of Claim 19 wherein the hematopoietic stem
    cells are human.

22. A method of Claim 21 wherein the hematopoietic stem
10  cells are glycophorin A$^{neg}$, CD38$^{low/neg}$, CD13$^{neg}$, CD15$^{neg}$,
    CD19$^{neg}$, CD20$^{neg}$, and CD34$^{neg}$.

23. A method of Claim 19 wherein the purified
    hematopoietic stem cells are obtained using the method
15  of purifying mammalian hematopoietic stem cells from a
    bone marrow cell sample comprising the steps of:
    a)  obtaining a mammalian bone marrow cell sample;
    b)  combining the cell sample of a) with a
        fluorescent, lipophilic vital dye which is a
20      substrate for a multiple drug resistant protein,
        under conditions appropriate for cells in the
        cell sample to take up the dye;
    c)  exposing the combination produced in b) to an
        excitation wavelength which causes fluorescence
25      of the dye;
    d)  assessing the fluorescence of the dye using an
        emission wavelength,
    wherein nucleated cells which contain the least amount
    of dye are purified mammalian hematopoietic stem
30  cells.

24. A method of Claim 19 wherein the purified
    hematopoietic stem cells are obtained using the method

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of purifying mammalian hematopoietic stem cells from a
bone marrow cell sample comprising the steps of:

a)    obtaining a mammalian bone marrow cell sample;

b)    combining the cell sample of a) with a
      fluorescent, lipophilic vital dye which is a
      substrate for a multiple drug resistant protein,
      under conditions appropriate for cells in the
      cell sample to take up the dye;

c)    exposing the combination produced in b) to an
      excitation wavelength which causes fluorescence
      of the dye;

d)    assessing the fluorescence of the dye using two
      different emission wavelengths simultaneously,

wherein nucleated cells which contain the least amount
of dye are purified mammalian hematopoietic stem
cells.

25.   A method of separating cycling hematopoietic stem
      cells from quiescent hematopoietic stem cells in a
      purified, mammalian hematopoietic stem cell sample
      comprising the steps of:

a)    obtaining a purified mammalian hematopoietic stem
      cell sample using the method of Claim 1;

b)    combining the cells of a) with Hoechst 33342 dye
      and verapamil; and

c)    measuring the amount of dye present in the
      hematopoietic stem cells using flow cytometry,

wherein purified hematopoietic stem cells which take
up the smallest amount of dye have a low concentration
of DNA and are designated as quiescent hematopoietic
stem cells, and purified hematopoietic stem cells
which take up a greater amount of dye have a greater
amount of DNA and are designated cycling hematopoietic
stem cells.

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26.  A method of Claim 25 wherein the hematopoietic stem cells are human.

27.  A method of identifying an agent which is a fluorescent, vital dye which is lipophilic for use in purifying mammalian hematopoietic stem cells comprising the steps of:

a)  obtaining a mammalian bone marrow cell sample;

b)  combining the cells of (a) with the agent under conditions appropriate for the cells to take up the agent;

c)  exposing the combination of (b) to an excitation wavelength which results in fluorescence of the agent;

d)  measuring the fluorescence at an emission wavelength,

wherein if distinct populations of nucleated cells are observed in which one of the population of cells takes up the smallest amount of dye and the population is purified, mammalian hematopoietic stem cells, then the agent is a fluorescent, vital dye which is lipophilic for use in purifying hematopoietic stem cells.

28.  A method of identifying an agent which is a fluorescent, vital dye which is lipophilic for use in purifying mammalian hematopoietic stem cells comprising the steps of:

a)  obtaining a mammalian bone marrow cell sample;

b)  combining the cells of (a) with the agent under conditions appropriate for the cells to take up the agent;

c)  exposing the combination of (b) to an excitation wavelength which results in fluorescence of the agent;

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d)   measuring the fluorescence using two emission
     wavelengths simultaneously,

wherein if distinct populations of nucleated cells are
observed in which one of the population of cells takes
up the smallest amount of dye and the population is
purified, mammalian hematopoietic stem cells, then the
agent is a fluorescent, vital dye which is lipophilic
for use in purifying hematopoietic stem cells.

29.   A method of transplanting bone marrow in a host
      comprising the step of introducing into the host the
      purified hematopoietic stem cells of Claim 1.

30.   A method of providing a host with purified
      hematopoietic stem cells comprising the step of
      introducing into the host the hematopoietic stem cells
      of Claim 1.

31.   A method of Claim 30 wherein the purified
      hematopoietic stem cells are cycling hematopoietic
      stem cells.

32.   A method of in vivo administration of a protein
      comprising the step of transfecting a purified
      hematopoietic stem cell produced by the method of
      Claim 19 with a construct comprising DNA or RNA which
      expresses a protein of interest.

33.   A method of Claim 32 wherein the purified
      hematopoietic stem cells are cycling hematopoietic
      stem cells.

34.   A method of Claim 30 wherein the purified
      hematopoietic stem cells are quiescent hematopoietic
      stem cells.

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35.     A method of Claim 32 wherein the purified
        hematopoietic stem cells are quiescent hematopoietic
        stem cells.

5   36.     Purified hematopoietic stem cells that are $CD34^{neg}$.

    37.     Purified hematopoietic stem cells of Claim 36 which
            are side population cells.

10  38.     Purified hematopoietic stem cells of Claim 37 which
            exhibit at least one of the following characteristics:
            glycophorin $A^{neg}$, $CD38^{low/neg}$, $CD13^{neg}$, $CD15^{neg}$, $CD19^{neg}$ and
            $CD20^{neg}$.

15  39.     Purified hematopoietic stem cells of Claim 36 which
            exhibit at least one of the following characteristics:
            glycophorin $A^{neg}$, $CD38^{low/neg}$, $CD13^{neg}$, $CD15^{neg}$, $CD19^{neg}$ and
            $CD20^{neg}$.

FIG. IA



FIG. IB

FIG. IC



FIG. ID
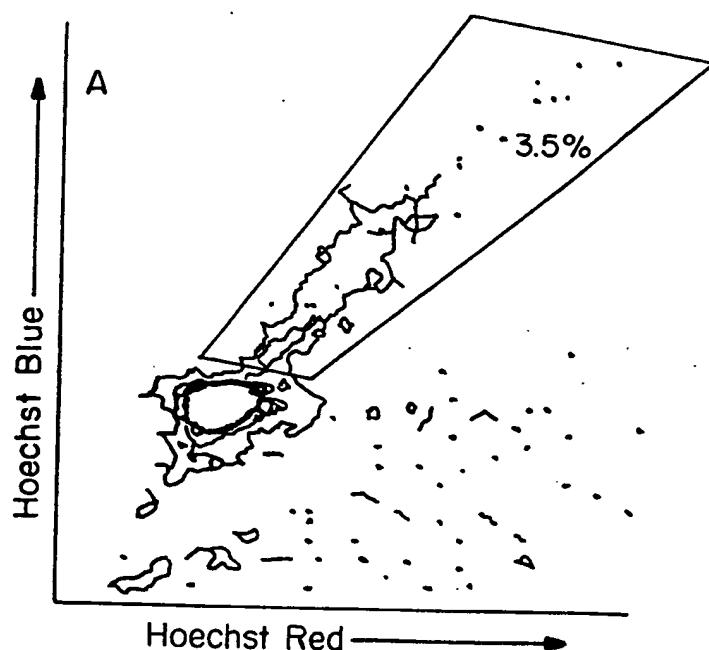
FIG. IE

Hoechst Red

# FIG. 2A



Hoechst Red
+ Verapamil
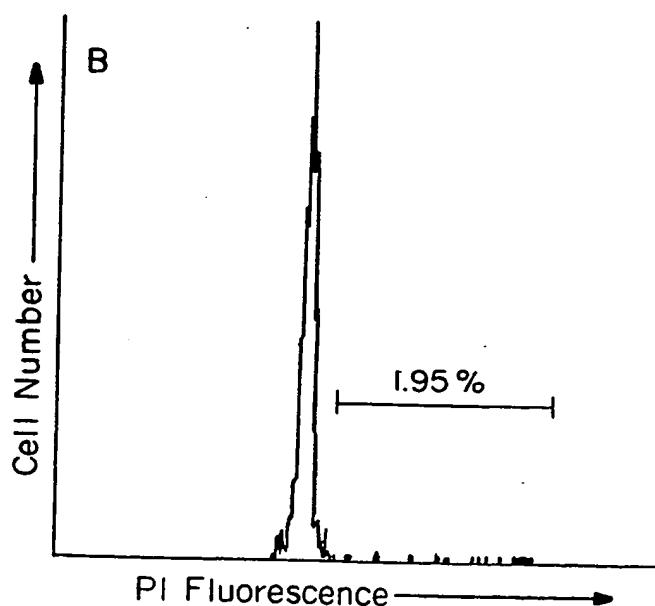
# FIG. 2B

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FIG. 3A

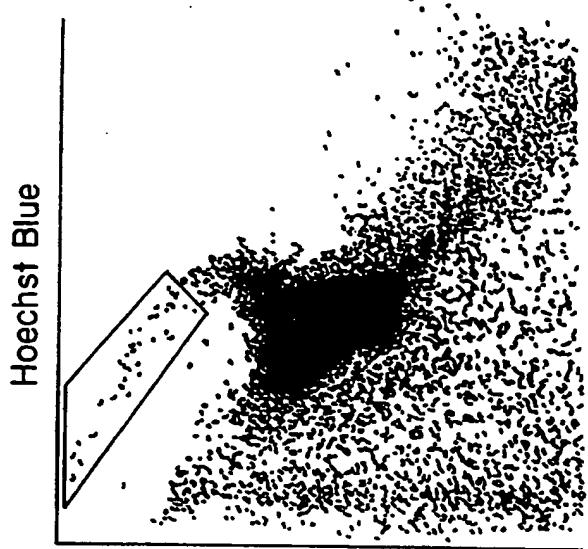

FIG. 3B

SUBSTITUTE SHEET (RULE 26)

Hoechst Red
Bone Marrow

## FIG. 4A
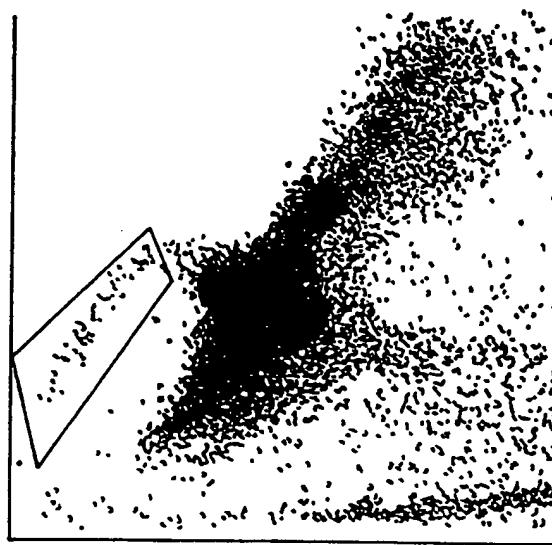


Cord Blood

## FIG. 4B

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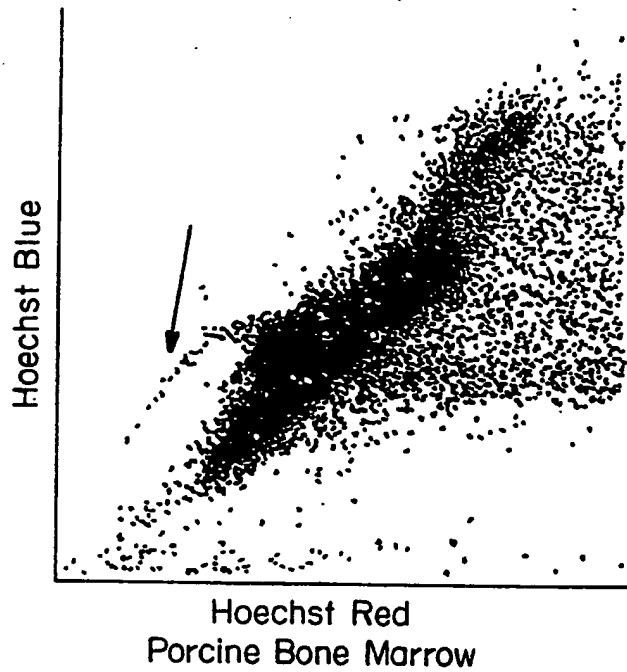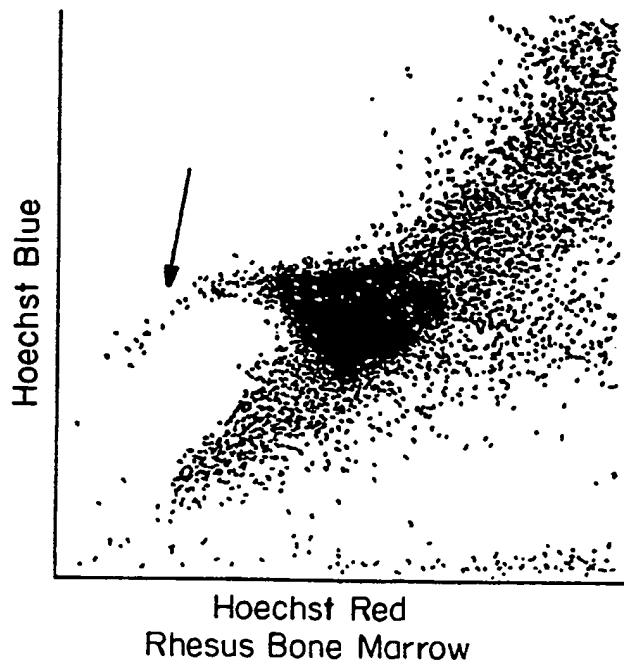


Hoechst Red
Porcine Bone Marrow

## FIG. 5A



Hoechst Red
Rhesus Bone Marrow

## FIG. 5B

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6    C12N5/08       C12Q1/24       C12Q1/02       A61K35/28       A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6    C12N    C12Q    A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | THE JOURNAL OF CELL BIOLOGY,<br>vol. 122, no. 4, August 1993, NEW YORK,<br>N.Y., US,<br>pages 897-902, XP000604719<br>W.H. FLEMING ET AL.: "FUNCTIONAL HETEROGENEITY IS ASSOCIATED WITH THE CELL CYCLE STATUS OF MURINE HEMATOPOIETIC STEM CELLS."<br>cited in the application<br>see page 897, abstract<br>see page 901, left-hand column, line 29 - line 33<br><br>---<br><br>-/-- | 1-39 |

[X] Further documents are listed in the continuation of box C.          [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 27 September 1996 | 1 4. 10. 96 |

| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Authorized officer<br><br>Ryckebosch, A |

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | EXPERIMENTAL HEMATOLOGY, vol. 21, no. 5, May 1993, LAWRENCE, KANS., US, pages 614-622, XP000604780 N.S. WOLF ET AL.: "IN VIVO AND IN VITRO CHARACTERIZATION OF LONG-TERM REPOPULATING PRIMITIVE HEMATOPOIETIC CELLS ISOLATED BY SEQUENTIAL HOECHST 33342-RHODAMINE 123 FACS SELECTION." see page 614, abstract see page 615, left-hand column, paragraph 6 - right-hand column, paragraph 2; figure 1 see page 621, left-hand column, paragraph 2 --- | 1-39 |
| A | CELL, vol. 66, 12 July 1991, CAMBRIDGE, MA, US, pages 85-94, XP002014549 P.M. CHAUDHARY ET AL.: "EXPRESSION AND ACTIVITY OF P-GLYCOPROTEIN, A MULTIDRUG EFFLUX PUMP, IN HUMAN HEMATOPOIETIC STEM CELLS." cited in the application see page 85, right-hand column, paragraph 2 - page 86, left-hand column, paragraph 2 --- | 1-39 |
| A | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, October 1990, WASHINGTON US, pages 7433-7437, XP002014550 G.J. SPANGRUDE ET AL.: "RESTING AND ACTIVATED SUBSETS OF MOUSE MULTIPOTENT HEMATOPOIETIC STEM CELLS." cited in the application see page 7433, abstract --- | 1-39 |
| A | EXPERIMENTAL HEMATOLOGY, vol. 18, no. 3, March 1990, LAWRENCE, KANS., US, pages 248-256, XP000604657 J.W.M. VISSER ET AL.: "PURIFICATION OF PLURIPOTENT HEMATOPOIETIC STEM CELLS: PAST AND PRESENT." see page 250, left-hand column, paragraph 4 - right-hand column, paragraph 3 --- -/-- | 1-39 |

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 183, no. 4, 1 April 1996, NEW YORK, N.Y., US, pages 1797-1806, XP000604752 M.A. GOODELL ET AL.: "ISOLATION AND FUNCTIONAL PROPERTIES OF MURINE HEMATOPOIETIC STEM CELLS THAT ARE REPLICATING IN VIVO." see the whole document ----- | 1-39 |

1

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims   29  -  35   are directed to a method of treatment

of the human/animal body the search has been carried out and based on the
alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest        [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)